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DIVERSITY ANALYSIS OF TOMATO GERMPLASM (*LYCOPERSICOM ESCULENTUM* MARKERS) USING SSR

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ABSTRACT

Study of phenotypic and genetic diversity in landrace collections is important for germplasm conservation. In addition, the characterization of much diversified materials with molecular markers offers a unique opportunity to define significant marker-trait associations of biological and agronomic interest. A collection of twenty four determinate and indeterminate cultivars of Tomato were screened with twenty SSR (simple sequence repeat) primers and four lycopene gene specific primers in order to determine genetic identities, genetic diversity and genetic relationships. On the basis of resolving power, primer T-45, T-62 and T-106 were most significant as they are able to recognize all 24 genotypes. The gene diversity was varied from 0.65 to 0.97 values with a mean diversity of 0.84. On an average, 54 scorable and reproducible alleles were amplified using all primers. UPGMA based dendrogram grouped the cultivars into two main cluster with two individual separated at one end of the dendrogram. Cluster analysis clearly showed that some genotypes are closely related while some are significantly distinct. The genetic distance information obtained in this study might be useful to breeder for planning the breeding programs.

KEYWORDS: Diversity, SSR, Microsattelites, Tomato

INTRODUCTION

Tomato (*Lycopersium esculentum*) is the second most economically important and consumed vegetable worldwide and a well-studied crop species in terms of genetics, genomics, and breeding. Tomato is a rich source of minerals, vitamins and organic acid, essential amino acids and dietary fibers. It is rich source of vitamin A and C and contains minerals like iron and phophorus. Tomato is rich source of minerals, vitamins, organic acid, essential amino acids and dietary fibres. Besides these tomato is an important source of antioxidant compounds viz. lycopene, β-carotene and polyphenols. Generally, all ripe tomato contains about 95% water, with the remaining 50% sugar (glucose and fructose), 15% acid (citric and malic) and 20-50mg/100g fruit weight of lycopene (Davies &Hobson, 1981). Intake of tomatoes and tomato-based products has been relatively consistently associated with a lower risk of a variety of cancers due to lycopene. Lycopene may alleviate chronic diseases such as cancer and coronary heart disease. Lycopene has also been found effective in the treatment of eye diseases, male infertility, inflammation, and osteoporosis. Experimental, clinical, and epidemiological studies have also established its role in the management of diabetes and hepatoprotection.

The traditional methodology of plant genetics based on morphological marker and biochemical marker for assessing the genetic diversity have been very effective in crop screening and improvement during the past several decades. This lengthy process may not allow the time sensitive need to increase the crop productivity in the future. An alternative approach for assessing the diversity among the cultivated genotypes is to use the molecular marker. With the advent of DNA marker technology in 1980s and 1990s, many limitations associated with morphological markers were

overcome and genetic mapping entered a new exciting and progressive era. DNA markers which are phenotypically neutral have allowed scanning of the whole genome in many crop plants including tomato.

Among crop species, tomato is very rich in the number of available molecular markers. Currently there are>1000 RFLP markers and approximately 214000 EST sequences. The use markers in tomato breeding have been limited by the lack of user-friendly markers such as SSRs (Simple Sequence Repeats). SSRs or microsatellites are PCR-based markers that have been developed in many plant species including tomato. SSR markers have the advantage of being multiallelic, highly polymorphic, co-dominant, and easily assayed in a basic laboratory set-up. However, especially in crops like tomato where genetic diversity is limited (Miller and Tanksley, 1990), the molecular marker of choice must be highly informative. For tomato, several primer sets for SSR analysis are available (Vosman *et al*., 2001, He *et al*., 2003). The utility of technique in tomato has been shown by Smulders *et al*., (1997), Bredemeijer *et al*., (2002), and Areshchenkiva and Ganal (1999). Study of phenotypic and genetic diversity in germplasm collection is important for germplasm conservation. In addition, the characterization of much diversified materials with molecular markers offers a unique opportunity to define significant marker trait association of biological and agronomic interest.

MATERIAL AND METHOD

Collection and Maintenance of Samples: Twenty four varieties of *L. escculentum* germplasm were collected from vegetable section of IIVR Varanasi. The seeds were sown for the preparation of nursery. Thereafter the individual plants were transferred into the pots and maintained in the Department of Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology Meerut.

Isolation of Genomic DNA: The fresh vegetative leaves were collected for the isolation of DNA. The genomic DNA was isolated by using CTAB following the standard protocol of Doyle & Doyle 1990 with some modification. 3 gm of leaf tissue was homogenized with liq- Nitrogen and transferred in centrifuge tube containing 15 ml of pre warmed (60°C) extraction buffer. The sample was mixed well and incubated for 1 hour in a shaking water bath at 65°C. The DNA was extracted with equal volume of C.I for 15 minutes and then centrifuges the sample at 8,000 rpm for 10 min. After centrifugation, aqueous phase is again extracted with equal volume of C.I. Aqueous layer was transferred to a fresh tube and DNA was precipitated with 1.5 ml of NaCl (5m). After this 5 µl of RNAs (10 mg/ml) was added to removes the RNA. For good precipitation of DNA, 0.6% volume of chilled isopropanal was added to the tube and incubated for 3 hours, then tubes were centrifuged at 10,000 rpm for 10 min. The pallet was washed with 5 ml of 70% ethanol, air dried and then dissolved in 100 µl of TE buffer.

PCR Reaction: A total of 20 SSR primers was used to study the genetic diversity of tomato germplasm. DNA amplification reaction was performed in 10 µl reaction volume which contained an end concentration 2.5 mM each of the dNTPs, 1 U/µl Taq polymerase enzyme, 25 ng DNA template and 1µl of reverse primer and 1µl of forward primer, 10 ng primer in Taq polymerase assay buffer (1 X) (10 X buffer contains 100 mM Tris-Cl, 500 mM KCl, 15 mM $MgCl_2$ and 0.1 % gelatin). Amplification reaction was carried out in a long -gene Thermal cycler with the following thermal profile: one cycle of 4 min at 94ºC (initial denaturation) followed by 45 cycles of 15 sec at 94ºC (denaturation), 45 sec (primer annealing at temp according to the primer sequence) and 90 sec at 72ºC (primer elongation), and finally one cycle of 4 min at 72ºC (final extension). Amplified PCR products were separated on 1.5 % (w/v) agarose gel in 1 X TBE (10 X TBE buffer contained 108 g tris base, 9.3 g EDTA and 55 g boric acid in 1 liter double distilled water, pH 8.3).

Electrophoresis was performed at 100 volt for 2 hr. Then the gel was visualized and photographed for further analysis. A low range DNA ruler (Bangalore Genei Pvt. Ltd.) was used as a molecular size marker. The reproducibility of the amplification products was checked twice for each polymorphic primer.

Analysis of SSR Marker Data: In order to assess the ability of primers to resolve the different varieties the resolving power (Rp) for each primer was calculated following **Prevost and Wilkinson's (1999)** method as Rp = Ib (band information). Resolving Power is calculated as 1-[2 x (0.5-p)], p being the proportion of the 06 varieties containing the bands and Gene Diversity is calculated as $1-\sum pi^2$ **(Anderson *et al*., 1993).**

The bands were scored as present (1) or absent (0) for each DNA sample with the all 20 SSR primer. Amplification was performed twice and only reproducible amplifications products were included in the data analysis. Similarity matrix using the similarity coefficient of **Jaccard (1908)** was constructed from the whole data. Pair wise distances between DNA accessions were calculated and analyzed using the Unweighted Pair Group Method Arithmetic average (UPGMA) **(Sneath and Sokal, 1973)**. Clusters were analyzed using the computer program NTSYS-PC, version 2.11s **(Rohlf, 2000)**. In some cases no band were observed, possibly due to insufficient homology between the primer and DNA template. There is also the possibility that this situation might have occurred by failure of the PCR caused by some other region as well.

RESULTS AND DISCUSSIONS

Collection and Maintenance of Tomato Germplasm: In order to assess the genetic diversity twenty four varieties of L. escculentum viz. Azad-1, TLBR-2, TLBR-5, PDT-3, PKM-1, Selection-7, Nandi, Pusa Rohini, Meghalaya local, Sikkim local, Pusa gaurav, Arkha abha, Punjab chhuhara, Castal Rock, KS-17, Money maker, Palam pink, IIHR-2200, IIHR-2201, Feb-2, H-86, FLA-7171, Tura local and Agata-30. Majority of genotypes germinate in 2-4 days. The growths of all plants were maintained in pots using conventional agricultural practices (Figure 1).

Isolation and Estimation of Genomic DNA: The genomic DNA from all 24 varieties of L. esculentum was isolated using the CTAB method. The quantity and quality of DNA was checked on 0.8% agarose gel by taking the standard molecular weight marker of 1Kb as reference at one end.

Molecular Profiling Using SSR Primers: To assess the genetic diversity of 24 genotypes of tomato, 20 SSR primers were used to amplify the genomic DNA of tomato (Figure 2). Out of 20 SSR primers used, 16 (80%) primers resulted in polymorphic, scorable and reproducible hence considered for the analysis. The number of polymorphic primers and fragments generated were not in similar range for all genotypes. Gene diversity was calculated for each primer, which varied from 0.65 to 0.97 values with a mean diversity of 0.84 (Table 1). Resolving power of the 16 SSR primers ranged from 0.78 to 3.82 with an average 1.91. In the present study, the SSR primers were not able to recognize all 24 genotypes and so could not be segregated on the basis of their ability to diagnose genotypes. Nevertheless, these could be ranked according to their Rp values under the reasonable premise that primers with higher Rp value have a greater capacity to separate different accessions (Prevost and Wilkinson, 1999).

Genetic Similarity Matrix and Cluster Analysis: The total of 20 primers amplified 57 allels across the 24 genotypes. The entire fragments generated in this way were polymorphic. Thus, the higher percent polymorphism suggests that the SSR procedure is a viable approach for the examination of genetic diversity of L. esculentum. The number of amplified allels observed ranged from minimum 2 to maximum 4 with primer-1/8/9 and primer-20

respectively. Based on the distance matrix expressed as similarity coefficient a dendrogram was generated by the UPGMA method. Similarity value for all the 24 accessions ranged from 0.38to 0.88 (Table 1).

The resultant dendrogram grouped the 24 genotypes into two main distinct clusters with two genotypes viz. Arkha Abha and H-86 did not grouped in any major cluster and placed outside the major clusters at one end of the dendrogram (Figure 3). The cluster 1 grouped 8 genotypes viz. Azad-1, TLBR-2, PDT-3-1, TLBR-5, PKM-1, Money maker Palam pink and Punjab Chhuahara. The main cluster 2 was subdivided into three sub clusters. The genotypes Agata-30 and PKM-1 showed significant genetic diversity with a coefficient value of 0.38. This showed that climatic conditions and physical parameters may affect the plant genome as the plant is adapted and these changes are inherited through genome to the next generation. The range of genetic diversity values broadly indicates the degree of heterogeneity or homogeneity in different genotypes of the plant species (Goswami and Ranade, 1999).Cluster analysis clearly indicates that Human intervention, which makes partitioning and distribution of variability complex is cited as reason for the grouping of different samples to one cluster suitable for different regions. The present study suggests that SSR is appropriate for analysis of genetic variability in closely related genotypes. Moreover, SSR could able to amplify the different loci of all the 24 genotypes.

CONCLUSIONS

For accessing the genetic diversity of genotypes of *Lycopersicum esculentum*, SSR primer 8, 13 and 19 were most significant as they are able to recognize all 24 genotypes and so could be segregated on the basis of their ability to diagnose genotypes. The estimating gene diversity was found most suitable for the purpose as they showed significant values. The present study suggests that primers used in this study were appropriate for the analysis of genetic variability in closely related genotypes. The SSR and Lycopene gene specific primers amplification profile for 24 genotypes of *Lycopersicum esculentum* showed that both categories of primers had excellent discriminating power and could accurately detect the structure of genetic diversity.

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APPENDICES

Table 1: Polymorphic SSR Markers and Corresponding Genetic Diversity and Resolving Power

S. No.	Primer Code	Sequence	GC (%)	Tm (^oC)	Genetic Diversity	Resolving Power
1	T-5	GACAAAAATTTTCCACACGGC (F) TCTCTTATAATTTTGTTGAGTCTCTGA(R)	40 45	53.0 52.0	0.65	1082
2	T-7	GTGGATTCACTTACCGTTACAAGTT(F) CATTCGTGGCATGAGATCAA (R)	32 37	51.5 52.4	0.89	1025
3	T-31	GGTAATACATTTTGAAGCTAAAAGC(F) TGGGAAGAACTCAAGTCAAAAA (R)	53 45	57.0 53.5	0.88	0.86
4	T-34	GTCAACTAGCGCTCCCAATCT (F) AAAGGGTTGTGGGAATTGTG (R)	44 35	53.2 53.1	0.82	0.78
5	T-38	GAATTAGAGGGTTTGTGATACCG (F) AAAAAGCTTCCTGGCTAAGAAAT (R)	48 40	55.0 52.0	0.75	1.55
6	T-40	GAGAGAGAGTATGTGCATTTCCC (F) TGAAAATTTGTGGTGTTGACG (R)	43 50	55.6 53.7	0.85	1.83
7	T-44	GCAACATTCCCAAGTCCAAAGA (F) TCGATGACCGCTAATAACCC (R)	43 40	54.2 53.6	0.88	1.66
8	T-45	GTGAGGGAGTGGGATTCAAAT (F) AATTAGGGGATACGGGATCG (R)	48 40	54.8 53.0	0.76	2.66
9	T-47	GTTGGAGTGCAATATTTGGGT (F) TTCGCTTAGGACAAGAATGACTT (R)	43 34	52.3 53.7	0.67	1.91
10	T-51	GTGAAGAGATGGGGTTGTGAA (F) TCTGTTTTGAAGGGAAGAAGATG (R)	46 48	56.1 53.7	0.79	2.29

Table 1: Contd.,

11	T-54	GAATCTTTGATGCGATTTCGG(F) TTTGTTAAAGTGTGTTAGCCGTTT (R)	40 45	51.8 53.7	0.88	2.00
12	T-57	GTGGACCATTTCAAGTTCAACA(F) TGAATGACATCCATCCATGA (R)	38 50	52.5 56.2	0.97	1.83
13	T-62	GTGACCACATGAGATATCCAGA(F) CAGTTGTCCATATTGTGTGGG (R)	48 48	55.6 53.8	0.89	2.72
14	T-64	CCAAAACCCAAGGAATAGCA(F) GGGAAATAAACCAAAATGAGAAGA (R)	30 38	50.3 53.2	0.87	1.99
15	T-70	AACATGCGGAGAAAAATT(F) GGAACACGTC CCAAAAATGT (R)	43 38	53.0 52.8	0.94	1.11
16	T-73	GTGAGGATTTTGATTTGTCTGAAG(F) TGGTTAGTTCTCCCTCACG (R)	30 36	51.4 55.3	0.85	1.99
17	T-103	GTGCGGATGGCTTTAAGGGACTG(F) CCTCTCAAAATCCTTGCCAC (R)	50 48	55.7 56.0	0.88	1.99
18	T-104	GTGAAAAATCACAAAACTTTGAAAAA(F) CGAAACACCCTCCATGTTAAA (R)	53 50	60.9 60.0	0.86	2.73
19	T-106	CCAAAACCCAAGGAATAGCA(F) GGGAGATAAACCAAAATGAGAAGA(R)	34 40	54.2 54.3	0.84	3.82
20	T-107	GCACAAATAATTTTTCAAGACCAA(F) AAAAACGGACATGTAGCTTTGTACT(R)	57 30	59.4 53.6	0.92	2.14

Similarity co-efficient matrix of 24 tomato genotypes

	Azad no-1	TLBR-2	PDT-3-1	TLBR-5	PKM-1	Selection -7	Nandi	PusaRohini	Meghalayal.ocal	Sikkim Local	Pusa Gaurav	Arkha Abha	PunjabChhuhara	Castal Rook	K.S-17	Money Maker	Palam Pink	IIHR-2200	IIHR-2201	02-Feb	H-86	FLA-7171	Tura Local	Agata-30
Azad no-1	0.1																							
TLBR -2	0.61	1																						
PDT-3-1	0.68	0.75	1																					
TLBR -5	0.68	0.75	0.77	1																				
PKM-1	0.63	0.75	0.72	0.72	1																			
Selection -7	0.63	0.61	0.59	0.77	0.5	1																		
Nandi	0.56	0.68	0.61	0.65	0.43	0.84	1																	
PusaRohini	0.59	0.7	0.63	0.72	0.54	0.81	0.84	1																
Meghalaya local	0.54	0.56	0.54	0.59	0.4	0.68	0.65	0.72	1															
Sikkim local	0.59	0.75	0.59	0.63	0.5	0.86	0.88	0.86	0.63	1														
PusaGaurav	0.63	0.65	0.63	0.77	0.54	0.81	0.79	0.86	0.59	0.81	1													
ArkhaAbha	0.61	0.59	0.61	0.56	0.56	0.61	0.63	0.56	0.47	0.65	0.7	1												
PunjabChhuhara	0.63	0.65	0.63	0.72	0.63	0.77	0.61	0.63	0.63	0.68	0.68	0.61	1											
Castal Rook	0.5	0.61	0.54	0.63	0.5	0.72	0.65	0.81	0.68	0.77	0.68	0.47	0.63	1										
K.S-17	0.52	0.72	0.52	0.56	0.56	0.56	0.59	0.75	0.65	0.7	0.61	0.5	0.56	0.79	1									
Money Maker	0.59	0.65	0.72	0.77	0.59	0.68	0.61	0.54	0.5	0.65	0.66	0.7	0.77	0.59	0.52	1								
Palam Pink	0.59	0.65	0.72	0.72	0.72	0.5	0.47	0.59	0.54	0.54	0.63	0.61	0.59	0.63	0.7	0.77	1							
IIHR-2200	0.56	0.63	0.52	0.7	0.52	0.75	0.63	0.7	0.52	0.75	0.75	0.5	0.56	0.79	0.68	0.7	0.65	1						
IIHR-2201	0.65	0.63	0.56	0.7	0.52	0.75	0.63	0.7	0.52	0.7	0.75	0.59	0.61	0.7	0.63	0.75	0.61	0.86	1					
Feb-02	0.59	0.65	0.59	0.72	0.59	0.72	0.61	0.77	0.63	0.72	0.72	0.52	0.63	0.86	0.79	0.72	0.77	0.88	0.84	1				
H-86	0.56	0.54	0.61	0.61	0.61	0.61	0.59	0.75	0.52	0.61	0.79	0.72	0.56	0.61	0.54	0.52	0.56	0.59	0.59	0.65	1			
FLA-7171	0.5	0.65	0.63	0.63	0.4	0.77	0.88	0.81	0.63	0.81	0.77	0.61	0.59	0.68	0.61	0.59	0.5	0.65	0.61	0.63	0.65	1		
Tura Local	0.47	0.68	0.61	0.7	0.56	0.75	0.77	0.75	0.61	0.79	0.7	0.5	0.65	0.79	0.63	0.7	0.65	0.77	0.72	0.75	0.5	75	1	
Agata-30	0.56	0.63	0.52	0.52	0.38	0.75	0.81	0.75	0.65	0.88	0.7	0.54	0.56	0.7	0.63	0.56	0.47	0.68	0.63	0.65	0.5	0.7	0.72	1

(A)

(B)

(C) (D) (E) (F)

Figure 1: Different Stages of Tomato Plant Maintaining for the Present Work

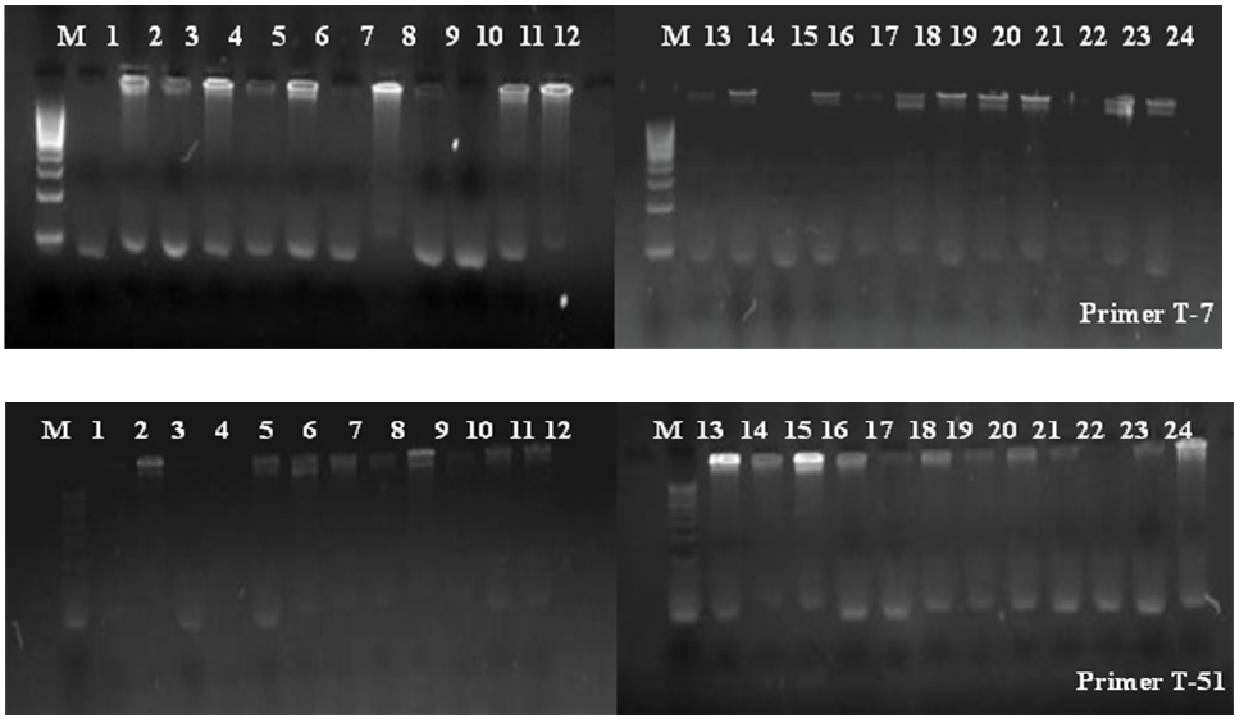

Figure 2: Amplification Profiling of 24 Genotypes of Tomato Using SSR Primers

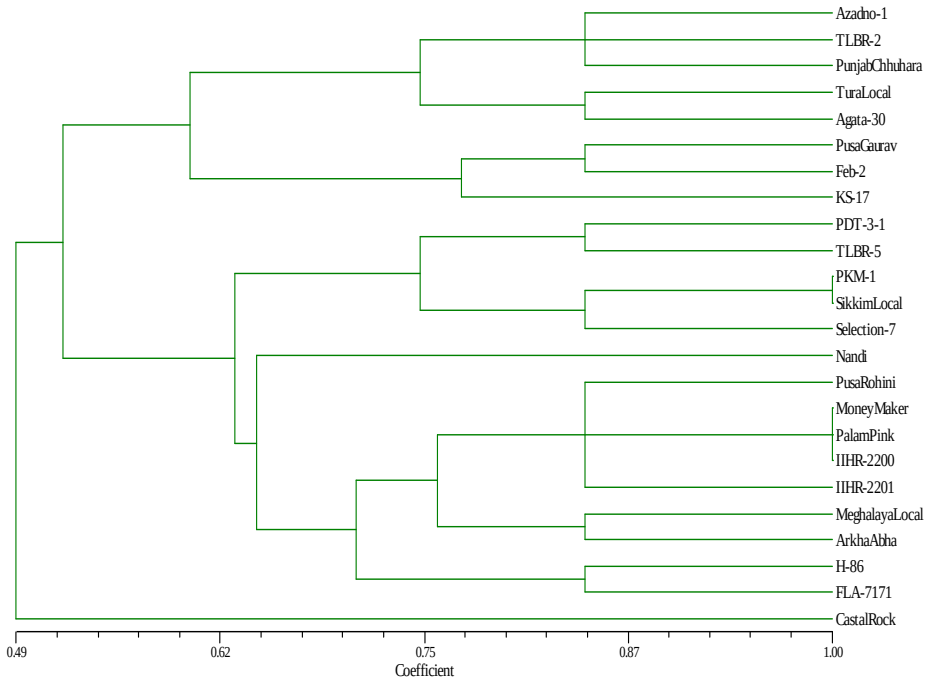

Figure 3: Grouping of 24 Genotypes of Tomato into Clusters Based on SSR Markers